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MODULATION BY BENZO[*a*]PYRENE OF EPIDERMAL GROWTH FACTOR RECEPTORS, CELL PROLIFERATION, AND SECRETION OF HUMAN CHORIONIC GONADOTROPIN IN HUMAN PLACENTAL CELL LINES

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Abstract—Clinical observations indicate that maternal cigarette smoking has significant detrimental effects on fetoplacental development. The present study used human trophoblastic choriocarcinoma cell lines of placental origin to investigate the effects of benzo[*a*]pyrene (BaP) on epidermal growth factor (EGF) receptors, cell proliferation and human chorionic gonadotropin (hCG) secretion. BaP decreased ^{125}I-EGF binding and EGF receptor protein in a concentration-related manner in both BeWo and JEG-3 cell lines. The steady-state level of EGF receptor mRNA, however, was not changed significantly by BaP in either cell line. Cell proliferation was unchanged or slightly increased following exposure to 10 and 50 μM BaP in the presence of serum, whereas proliferation progressively decreased in cells exposed under serum-free conditions. The mitogenic effect of EGF was inhibited by cotreatment with BaP in both cell lines. Further study of trophoblast endocrine function showed that both basal and EGF-stimulated secretion of hCG was reduced significantly by BaP exposure in BeWo cells, whereas no adverse effect was seen in JEG-3 cells. Finally, cytochrome P450 1A1 (CYP1A1) was induced in a concentration-dependent manner by BaP in both cell lines. Thus, data indicate that the BaP-mediated loss of EGF receptors alters trophoblast proliferation and endocrine function, and that different mechanisms may be involved in the regulation of hCG secretion in BeWo and JEG-3 cells. In addition, this study supports the feasibility of using the BeWo and JEG-3 trophoblastic choriocarcinoma cell lines to investigate biomarkers and mechanisms of placental toxicity.

Key words: BaP; EGF receptor; cell proliferation; hCG secretion; CYP1A1; choriocarcinoma cells

EGF§ receptor is a well-characterized 170 kDa single polypeptide transmembrane glycoprotein, which is detectable in a wide variety of tissues *in vivo* and in cell lines in culture [1]. Human placenta shows a high level of expression of EGF receptor, which is localized in the proliferative cytotrophoblasts in very early placenta, and subsequently in mitotically inactive differentiated syncytiotrophoblasts as gestation advances [2]. The physiologically important ligands EGF and TGF-α are also expressed in trophoblasts throughout gestation [2, 3]. EGF and TGF-α have been shown to stimulate trophoblast proliferation [3–5], and EGF stimulates secretion of the peptide hormone hPL and hCG [4, 6]. Studies with human placental trophoblastic choriocarcinoma cells also show that EGF can stimulate hCG secretion [7], and the growth of transplanted choriocarcinoma cells is altered by EGF in association with changes in EGF receptor binding activity and mRNA level [8]. Thus, the evidence strongly supports a physiological role for the EGF receptor-EGF/TGF-α system in normal fetoplacental growth and development throughout pregnancy.

Maternal cigarette smoking during pregnancy has been associated with spontaneous abortion and fetal growth retardation [9, 10]. Study of placentas from cigarette smokers and nonsmokers found that EGF-stimulated receptor kinase activity is decreased markedly in placental membrane proteins from smokers [11, 12]. In this regard, BaP is a potent polycyclic carcinogen that is present in the particulate phase of cigarette smoke [13]. Our study of human placental cells in primary culture found that exposure to BaP directly resulted in a concentration-dependent selective loss of EGF receptor binding activity and autophosphorylation, which was greatest in cells from first trimester placentas [14]. It is significant that the original observation of selective alterations in EGF, but not insulin, receptors in the placentas of women who smoked [12] was confirmed recently by Gabriel *et al.* [15], and further shown to be linked with intrauterine growth retardation in these pregnancies. A separate study of women who were exposed to polychlorinated biphenyl-contaminated rice oil found that birth weights are decreased in infants following *in utero* exposure in association with decreased placental EGF receptor tyrosine kinase activity [11, 16]. These and other reports [17, 18] provide substantial evidence that EGF receptors are altered in placental membranes from women whose fetuses show intrauterine growth retardation.

The present study was undertaken to examine mech-

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§ Abbreviations: EGF, epidermal growth factor; BaP, benzo[*a*]pyrene; hCG, human chorionic gonadotropin; CYP1A1, cytochrome P450 1A1; TGF-α, transforming growth factor-α; hPL, human placental lactogen; K_d, dissociation constant; B_{max}, maximum binding capacity; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Ah, aryl hydrocarbon; and SSC, 0.015 M NaCl + 0.015 M sodium citrate.

anisms by which exposure to the prototype environmental carcinogen BaP may alter placental trophoblast growth and endocrine function. Further investigation of the cellular consequences of a BaP-mediated loss of EGF receptors has been complicated by the fact that placental trophoblasts do not actively proliferate in primary culture [19]. For this reason, we chose to study BeWo and JEG-3 human trophoblastic choriocarcinoma cell lines as a model of proliferative early trophoblast, which secretes the major placental peptide hormone hCG [19, 20]. The results indicate that BaP exposure is associated with a loss of EGF receptors, altered cell proliferation, and hormone secretion in the choriocarcinoma cells, changes that are significant potential mechanisms of placental toxicity.

MATERIALS AND METHODS

Materials

Recombinant human EGF was purchased from Genzyme (Cambridge, MA). BaP was obtained from the Sigma Chemical Co. (St. Louis, MO) and stored in the dark. The polyclonal sheep anti-human EGF receptor antiserum was from Upstate Biotechnology, Inc. (Lake Placid, NY), and the polyclonal goat anti-rat CYP1A1 antiserum from Gentest (Woburn, MA); the Vectastain® ABC kit (containing the rabbit biotinylated anti-sheep IgG) and horse peroxidase-labeled anti-goat IgG were from Vector Laboratories Inc. (Burlingame, CA). ^{125}I-Protein A and [α-^{32}P]dCTP were obtained from ICN (Irvine, CA), and ^{125}I-EGF from Amersham (Arlington Heights, IL). The plasmid containing cDNA for human EGF receptor (pE7) was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The prime-a-gene® labeling system, *Cla*I restriction enzyme and CellTiter 96™ non-radioactive cell proliferation assay kit were purchased from Promega (Madison, WI). The Micro-Elisa total βeta-hCG test kit was purchased from Leinco Technologies Inc. (St. Louis, MO), and cell culture media and fetal bovine serum (FBS) were from Gibco/BRL (Grand Island, NY). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

Methods

Cell cultures. The human epidermoid carcinoma cell line A-431 and the human choriocarcinoma cell lines BeWo and JEG-3 were obtained from the ATCC. A-431 cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose and 10% FBS, BeWo cells in Ham's F-12 medium supplemented with 15% FBS, and JEG-3 cells in Eagle's minimum essential medium supplemented with 10% FBS, respectively, in a humidified atmosphere containing 5% CO_2 at 37°. All complete media were supplemented with penicillin (100 μg/mL), streptomycin (100 μg/mL) and amphotericin B (2.5 μg/mL). Cells were grown to confluency and media changed every 2–3 days. Confluent cells were subcultured after trypsinization.

Chemical treatment. Twenty to forty-eight hours after subculture of BeWo and JEG-3 cells, the medium was replaced and various concentrations of BaP were added in the presence or absence of serum [14]. Stock solutions of BaP were prepared in DMSO, with the final concentrations of BaP in media being 1 nM to 50 μM in 0.1% DMSO. Control cultures received 0.1% DMSO. EGF receptor protein and mRNA, cell proliferation, and hCG concentrations were then determined at the indicated times. For EGF stimulation of hCG secretion, cells were placed in serum-free medium with or without BaP 24 hr prior to EGF addition. After three washes with Hanks' solution, cells were exposed to EGF in serum-free medium for another 24 hr. The hCG concentration in the medium was then determined. Each set of cultures was carried out in triplicate.

EGF binding assay. Cells were washed thoroughly to remove BaP and then incubated with 100 pM ^{125}I-EGF in the presence or absence of unlabeled EGF for 1.5 to 2 hr at room temperature or for 5 hr at 4°. After careful rinsing to remove unbound radioactivity, the cells were solubilized, and the total binding of ^{125}I-EGF was determined by gamma counting. Specific binding was expressed as the difference between radioactivity bound in the absence (total binding) and presence (nonspecific binding) of excess unlabeled EGF (100 nM). For Scatchard analysis, cells were incubated with increasing concentrations of ^{125}I-EGF (1.25 to 200 pM) for 1.5 hr at room temperature. Nonspecific binding of ligand was measured by adding excess unlabeled EGF to cultures for each concentration of ^{125}I-EGF. Each point on the Scatchard plot represents specifically bound ^{125}I-EGF.

Western immunoblot analysis. Cells were rinsed three times, collected by scraping with a rubber policeman, and lysed in 1 mL PBS using three freeze-thaw cycles. The total cell membrane fraction as obtained by centrifugation at 12,000 *g* for 10 min at 4° and resuspended in PBS. Samples of A-431 membrane protein (50 μg) and BeWo and JEG-3 membrane protein (100 μg) were then separated by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose filters using 25 mM Tris, 192 mM glycine buffer at pH 8.2, according to the procedure of Towbin *et al.* [21]. The blotted nitrocellulose filters were washed in 100 mM Tris containing 0.1% (v/v) Tween 20 and 0.9% NaCl, pH 7.5 (TTBS) for 30 min, and then incubated sequentially with polyclonal anti-human EGF receptor antiserum (diluted to 1 μg/mL in TTBS) or preimmune sheep serum for 60 min, followed by biotinylated anti-sheep IgG for 60 min, and Vectastain® ABC reagent for 30 min. Immunoreactive bands were visualized by incubation with 3-amino-9-ethylcarbazole in the presence of 0.015% hydrogen peroxide. Quantitation of immunoreactive EGF receptor protein was carried out by a modification of the method of Gargosky *et al.* [22]. In brief, nitrocellulose filters were blocked with 1% (w/v) BSA in TTBS for 18 hr at 4°, and then incubated sequentially with sheep anti-EGF receptor antiserum or preimmune sheep serum, rabbit anti-sheep IgG, and ^{125}I-protein A. The immunoreactive bands were quantitated by scanning cpm using a βeta-scope 603 blot analyzer, after which filters were exposed to X Omat film at −80° for 12–18 hr for autoradiography.

For determination of CYP1A1, 100 μg protein of the whole cell lysate was separated by 10% SDS-PAGE, electrophoretically transferred, and immunostained with goat anti-rat CYP1A1 (1:500 dilution) as described previously [12].

RNA isolation and northern blot analysis. Total cellular RNA was isolated from cultured cells by acid guanidinium thiocyanate phenol-chloroform extraction according to Xie and Rothblum [23]. For northern blotting, 40 μg of total cellular RNA was denatured, fractionated in 1% agarose formaldehyde gel, and trans-

ferred to nitrocellulose or nylon membranes. A 2.4 kb *ClaI* digested fragment of the EGF receptor cDNA was used as a probe. The probe was labeled with [α-^{32}P]dCTP using a random primer DNA labeling kit. Prehybridization was carried out in 50% formamide containing 5× Denhardt's solution, 4× SSC, 0.1% (w/v) SDS, 40 mM sodium phosphate, and 0.25 mg/mL yeast RNA at 42° overnight. The hybridization was performed at 42° for 40 hr in the same buffer but containing 1× Denhardt's solution and the ^{32}P-probe. The filter was washed twice in 2× SSC/0.1% SDS at room temperature for 30 min, then twice in 0.2× SSC/0.1% SDS at 42° for 30 min, and once at 0.1× SSC/0.1% SDS at 65° for 15 min. Transcripts were visualized by autoradiography for 5–7 days at −80°. After removing the EGF receptor probe, the filter was rehybridized with ^{32}P-labeled β-actin cDNA. Hybridization signals were quantitated by densitometric scanning with the EGF receptor message standardized to the β-actin transcript.

Cell proliferation assay. Cell proliferation was determined using the tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT dye assay according to the CellTiter 96™ non-radioactive cell proliferation assay procedure (Promega). This assay is based on the cellular conversion of the tetrazolium salt into a formazan product that is quantitated using an ELISA plate reader. Direct comparisons between [^{3}H]thymidine incorporation and the MTT assay have shown less than a 5% difference between assays for determination of growth-factor concentrations. Briefly, 20 hr after subculture in 96-well tissue culture plates at a density of 5.0×10^3/well for BeWo and 2.5×10^3/well for JEG-3, cells were exposed to various concentrations of BaP and/or EGF for 48 hr in the presence or absence of FBS, followed by a 4-hr incubation with MTT dye. After solubilization of the formazan product, the extinction at 595 nm was recorded with 690 nm as reference wavelength using an ELISA plate reader. Relative cell proliferation was determined by comparing the extinction with that of control cells.

hCG assay. Assay of hCG in the media was carried out with a Micro-Elisa total βeta-hCG test kit (Leinco Technologies, Inc.). Briefly, the test sample is allowed to react simultaneously with the coated and conjugated antibodies, resulting in the hCG molecule being sandwiched between the solid phase and enzyme-linked antibodies. After a 30-min incubation at room temperature, the sample well is washed thoroughly to remove free enzyme-labeled antibody. An enzyme substrate-chromogen is added to the well and incubated for 15 min at room temperature, resulting in the development of a blue color. The addition of 1.0 N H_2SO_4 converts the color to yellow. The extinction at 450 nm is then recorded, which is directly proportional to the concentration of hCG in the sample.

Protein assays. Protein concentration was determined by the method of Bradford [24] using BSA as a standard.

Data analysis. All experiments were performed using triplicate or quadruplicate cultures at each concentration of EGF, BaP and/or time point. One-factor ANOVA was employed to assess concentration effects followed with Fisher's protected least significant difference (PLSD) test when a significant concentration effect ($P < 0.05$) was detected in the ANOVA. A two-factor ANOVA was employed to analyze the BaP concentration effect, the EGF treatment effect and the potential interactive effect on cell proliferation. An unpaired Student's *t*-test was also used to analyze the data. All statistical analyses were performed with the Macintosh StatView512+™ program.

RESULTS

Effects of BaP on specific binding of ^{125}I-EGF and immunoreactive receptor protein

Exposure to BaP for 48 hr significantly reduced the specific binding of ^{125}I-EGF to BeWo and JEG-3 cells in a concentration-related manner (Fig. 1). Specific ligand binding to BeWo cells was inhibited significantly by 33 and 61% at 1 and 50 μM BaP, respectively, while binding to JEG-3 cells was reduced significantly by 23 and 63%, respectively. A representative Scatchard plot of EGF binding to JEG-3 cells is shown in Fig. 2. Scatchard analysis showed a single class of binding sites with a K_d of 0.043 nM for BeWo and 0.048 nM for JEG-3 cells (Fig. 2), which is similar to the high-affinity binding site previously reported in human placental cells [14] and human placental membranes [11]. Cultures treated with 10 μM BaP for 48 hr exhibited 34 and 42% decreases in the B_{max} in BeWo and JEG-3 cells, respectively, with little change in K_d values (Fig. 2).

Furthermore, comparable reductions in ^{125}I-EGF-specific binding were observed when binding was measured at 4° or room temperature (Table 1). Internalization of the EGF receptor in choriocarcinoma cells does not occur at 4°, insofar as more than 90% of the ^{125}I-EGF bound to both control and BaP-treated BeWo and JEG-3 cells was dissociated by an acid wash procedure that removes surface bound ligand. Therefore, these data indicate that the BaP-related decrease in EGF binding is

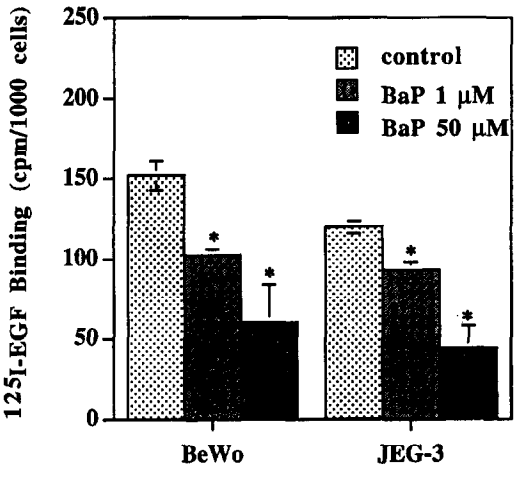

Fig. 1. BaP effects on specific binding of ^{125}I-EGF to BeWo and JEG-3 cells. The BeWo and JEG-3 cells (5×10^4 cells/well) were incubated with 0, 1 and 50 μM BaP in the presence of FBS for 48 hr. The cells were washed and incubated with 100 pM ^{125}I-EGF in the absence or presence of unlabeled 100 nM EGF for 2 hr at room temperature. After careful rinsing to remove unbound radioactivity, the cells were solubilized, and the total binding of ^{125}I-EGF was determined by gamma counting. Specific binding was determined as the difference between radioactivity bound in the absence (total binding) and presence (non-specific binding) of excess unlabeled EGF. Values are the means ± SEM of triplicate cultures. Key: (*) differs from control at $P < 0.05$ by Fisher PLSD and unpaired Student's *t*-test.

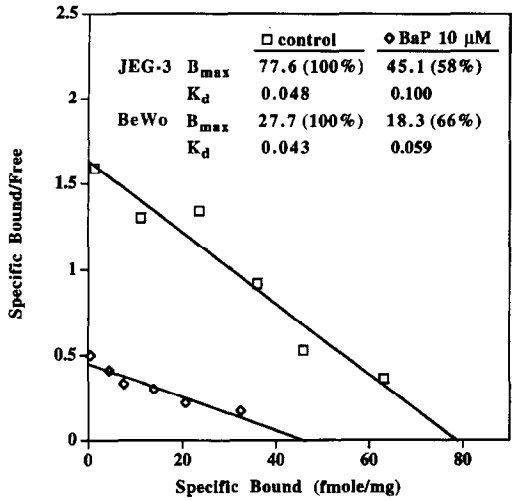

Fig. 2. Scatchard plot analysis of ^{125}I-EGF binding to control and BaP-treated JEG-3 cells. Cells were treated with 0.1% DMSO (control) or 10 μM BaP for 48 hr prior to incubation with increasing concentrations of ^{125}I-EGF (1.25 to 200 pM) at room temperature for 1.5 hr in the presence or absence of excess unlabeled EGF. Specific binding was determined as described under Materials and Methods and expressed as femtomoles ^{125}I-EGF bound per milligram of total cell protein. B_{max} (fmol/mg) and K_d (nM) values for ^{125}I-EGF binding to BeWo and JEG-3 cells were determined by Scatchard analysis of equilibrium binding data.

due to an alteration in available high-affinity cell surface binding sites rather than internalization of the ligand.

EGF receptor protein in choriocarcinoma cells was initially detected by avidin-biotin immunoperoxidase techniques with a polyclonal anti-human EGF receptor antibody. Consistent with our previous observations in human placentas [12], the native EGF receptor was detected as major 150–170 kDa bands in the total cell membrane fraction of both BeWo and JEG-3 cells (Fig. 3A), which was comparable to the receptor species detected in human A-431 cells and rat liver microsomes (data not shown). Exposure of BeWo and JEG-3 cells to BaP for 48 hr showed a concentration-related decrease in levels of total cell EGF receptor immunoreactive protein in both cell lines (Fig. 3A). Quantitation of the cross-reactive bands using iodinated-protein A revealed that the immunoreactive EGF receptor protein was decreased from control by 20, 41 and 52% in BeWo cells, and by 23, 58 and 53% in JEG-3 cells, following exposure to 1, 10 and 50 μM BaP for 48 hr, respectively (Fig. 3B). No lower molecular weight immunoreactive receptor species were detected in association with the decrease in native 150–170 kDa receptor protein following BaP treatment. These data provide further evidence that the reduced ^{125}I-EGF cell surface binding was due to the loss of total cellular high molecular weight EGF receptor protein.

Effect of BaP on EGF receptor mRNA levels

The effect of BaP on steady-state levels of EGF receptor mRNA was analyzed by northern blot techniques using a pE7 EGF receptor cDNA probe. Consistent with previous reports [25], multiple transcripts of 10, 5.6, 4.6 and 2.9 kb appeared in reference A-431 cells (Fig. 4A). BeWo and JEG-3 cells both showed a major transcript of 10 kb and two minor species of 5.6 and 4.6 kb. Quantitation of these bands further showed that the steady-state level of EGF receptor mRNA was not changed significantly by BaP treatment in either cell line (Fig. 4B). These data suggest that the BaP-mediated decrease in EGF receptor protein does not involve changes in steady-state levels of mRNA.

Effects of BaP on cell proliferation

Cell proliferation assays were conducted to determine whether BaP treatment was associated with cytotoxic effects on cell division. Data in Fig. 5 show that, under serum-free conditions, BeWo and JEG-3 cell proliferation was inhibited 30–50% with BaP at 10 and 50 μM. In contrast, exposure to BaP over a concentration range of 1 nM to 50 μM had no significant effect on cell proliferation up to 48 hr in BeWo and JEG-3 cells cultured in the presence of serum. Thus, BaP at higher concentrations adversely affected cell viability only under serum-free conditions.

We next examined whether BaP treatment altered EGF-stimulated cell proliferation. EGF at concentrations of 100 (16 nM) and 200 ng/mL significantly stimulated cell proliferation 1.5- to 2-fold under serum-free conditions in both cell lines (Fig. 6). When cells were exposed to EGF (100 ng/mL) and BaP together for 48 hr, the EGF stimulation of cell proliferation was still observed at 10 μM BaP, but was decreased significantly from control at 50 μM BaP in both cell lines. A two-factor ANOVA, however, indicates that there was no significant interactive effect between BaP and EGF on BeWo cell proliferation. In contrast, there was a significant interactive effect of BaP and EGF on JEG-3 cell proliferation ($P < 0.05$). In this regard, a higher concentration of EGF (200 ng/mL) protected JEG-3 cells, but not BeWo cells, from the loss of EGF stimulation of cell proliferation in the presence of 50 μM BaP. This protective effect of high concentration EGF in JEG-3 cells was similar to the protective effect of serum on maintaining cell viability in the presence of 50 μM BaP (Fig. 5).

Effect of BaP on basal and EGF-stimulated hCG secretion

The next experiment evaluated whether BaP exposure was associated with altered trophoblast endocrine function as measured by secretion of hCG. Data in Fig. 7 show that BaP treatment for 48 hr in the presence of serum inhibited basal hCG secretion by BeWo cells, but not by JEG-3 cells. The hCG concentration in the BeWo cell media was reduced significantly by 41, 56 and 64% at 1, 10 and 50 μM BaP after 48 hr of exposure, respectively, which correlates closely with decreased EGF receptor protein (Fig. 3). However, the level of basal hCG secretion by treated JEG-3 cells remained at a high level, as in control cells.

To assess whether BaP can inhibit EGF-stimulated hCG secretion, the cells were first incubated with BaP for 48 hr, and then treated with EGF for another 24 hr in serum-free medium. As shown in Fig. 8, EGF stimulated hCG secretion 3-fold in untreated control BeWo cells; the response, however, was decreased significantly by 29 and 43% in BeWo cells pretreated with BaP at 1 and 10 μM, respectively. In contrast, in JEG-3 cells, BaP pretreatment did not alter the stimulation of hCG secretion by EGF, with EGF at 100 ng/mL producing an approximate 2-fold increase in all three groups of JEG-3 cells. Thus, differential effects of BaP were observed on hCG secretion by BeWo and JEG-3 cells.

Table 1. Effect of temperature on inhibition of ^{125}I-EGF binding by BaP in BeWo and JEG-3 cells

Cell	Treatment	^{125}I-EGF binding (cpm/μg) 24°	4°
BeWo	Control	46.9 ± 1.9 (100%)	16.7 ± 0.38 (100%)
	BaP (10 μM)	28.1 ± 2.9* (60%)	12.0 ± 0.67* (72%)
JEG-3	Control	54.0 ± 4.2 (100%)	38.3 ± 1.8 (100%)
	BaP (10 μM)	31.2 ± 0.6* (58%)	22.3 ± 1.2* (58%)

The cells were treated with 0.1% DMSO (control) or 10 μM BaP for 48 hr prior to incubation at room temperature (24°) for 1.5 hr or at 4° for 5 hr with 100 pM ^{125}I-EGF in the presence or absence of unlabeled 100 nM EGF. The specific binding was normalized with respect to protein in each sample and expressed as counts per minute per microgram protein of total cell lysate. Values are the means ± SEM of triplicate cultures.
* Differs from control at $P < 0.05$ by unpaired Student's t-test.

Induction of cytochrome P4501A1

A final experiment examined whether CYP1A1 was induced by BaP under experimental conditions in the choriocarcinoma cells. Figure 9 shows that treatment with BaP at 1, 10 and 50 μM for 48 hr markedly increased cellular CYP1A1 content in a concentration-related manner in both cell lines. Insofar as 100 μg of cell membrane protein was applied to each lane, the intensities of the respective lanes indicate that more immunoreactive 55 kDa CYP1A1 protein was present in JEG-3 cells, compared with BeWo cells following BaP exposure. In addition, induction of CYP1A1 is a sensitive marker for exposure to 1 μM BaP in these cells.

DISCUSSION

Maternal cigarette smoking during pregnancy has been associated with miscarriage, fetal growth retardation and increased perinatal mortality [9, 10]. Changes in placental morphology have been reported in association with maternal smoking [26], as have alterations in amino acid uptake, hPL secretion [27, 28] and a selective loss of placental EGF receptor autophosphorylation [11, 12, 15]. A major carcinogen present in the particulate matter of cigarette smoke is BaP, an inducer of CYP1A1 [13, 29]. Exposure of cultured cells from early gestation human placentas to BaP was found previously to directly produce a selective concentration-dependent loss of EGF receptor binding and autophosphorylation [14]. The original observations on selective alterations in EGF, but not insulin receptors in placentas from smokers [12] have been confirmed recently by Gabriel *et al.* [15] and further shown to be correlated with intrauterine growth retardation in these pregnancies. Thus, smoking-related changes in EGF receptor may be mediated via BaP and may underlie a number of the biochemical and morphological alterations observed in placentas from women who smoke. However, further investigation of the effects of a loss of EGF receptors on trophoblast growth and function has been difficult because placental trophoblasts in primary culture do not actively proliferate [19]. Further study of the mechanism of placental toxicity of BaP needs a model of proliferative trophoblasts, and we chose to evaluate two human choriocarcinoma cell lines, BeWo and JEG-3, the latter being more proliferative in lower concentrations of serum. Both cell lines retain many characteristics of normal human trophoblast cells, produce similar steroid and peptide hormones such as

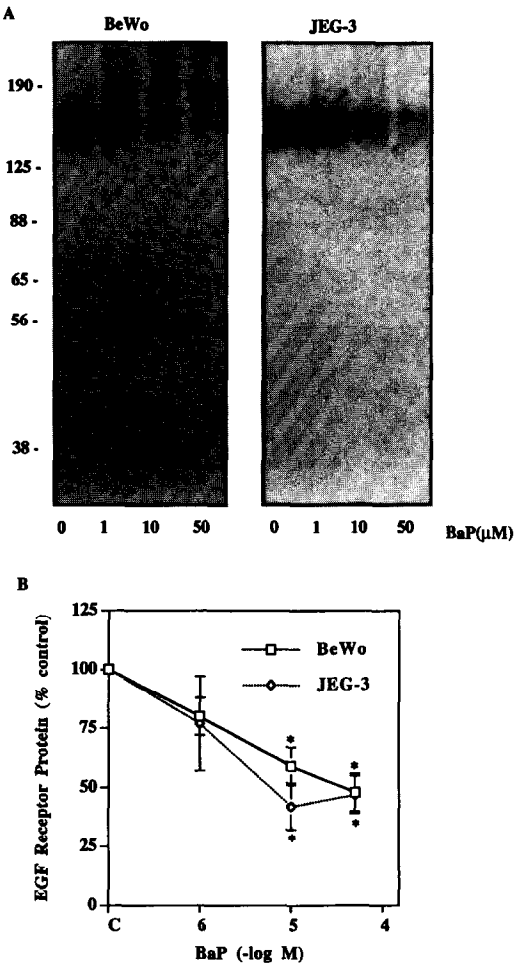

Fig. 3. BaP effects on immunoreactive EGF receptor protein in BeWo and JEG-3 cells. The cells were harvested following incubation with BaP for 48 hr in the presence of serum. Cell membrane protein (100 μg) was electrophoresed, transferred, and probed with sequential sheep anti-EGF receptor, rabbit anti-sheep IgG and ^{125}I-protein A as described in Materials and Methods. The immunoreactive proteins were then evaluated by (A) autoradiography, and (B) quantitation of the intensities of the 150–170 kDa bands by βetascope scanning, with the average cpm of the controls (C) being arbitrarily set as 100%. Values are the means ± SEM of three separate experiments. Key: (*) differs from control at $P < 0.05$ by Fisher PLSD and unpaired Student's t-test.

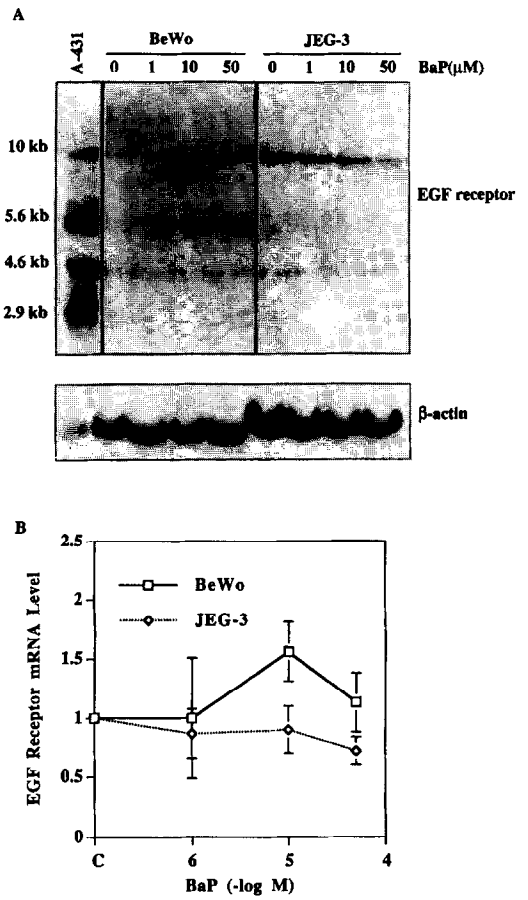

Fig. 4. Steady-state levels of EGF receptor mRNA in BeWo and JEG-3 cells following culture in the absence or presence of BaP for 48 hr. Total RNA (40 μg) was denatured, blotted, hybridized with the ^{32}P-labeled EGF receptor cDNA probe, and subsequently rehybridized with the ^{32}P-labeled β-actin cDNA as described in Materials and Methods (A) Autoradiograms of the northern blots. (B) Quantitation of the 10, 5.6 and 4.6 kb transcripts of the EGF receptor mRNA, with the ratio of EGF receptor message to β-actin message in the control cells (C) being set as 1. Values are the means ± SEM of three separate experiments.

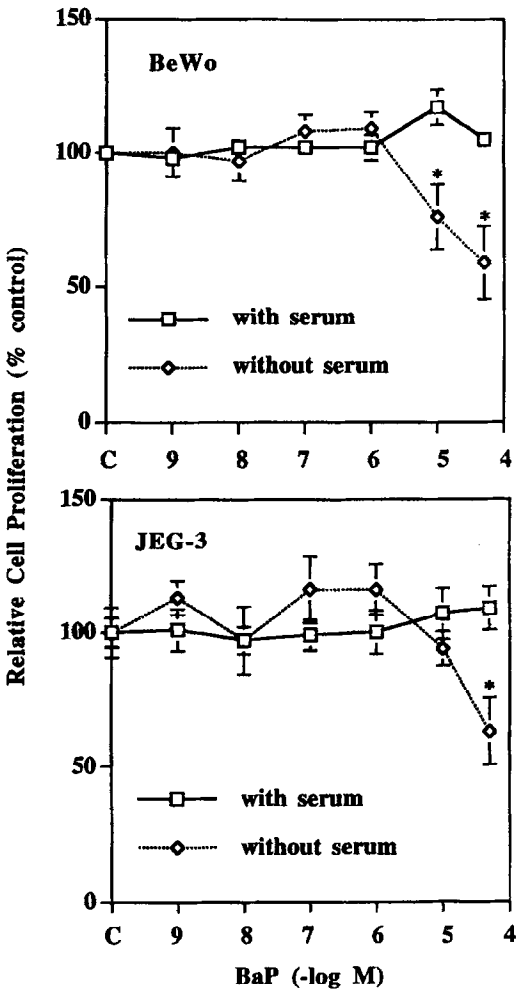

Fig. 5. Effects of BaP on BeWo and JEG-3 cell proliferation in the presence or absence of FBS. BeWo and JEG-3 cells were cultured at densities of 5×10^3 and 2.5×10^3 cells/well, respectively, for 20 hr and treated with various concentrations of BaP for another 48 hr in the presence or absence of FBS, respectively. Relative cell proliferation was determined by the nonisotopic MTT assay. Proliferation of the control cells (C) was arbitrarily set as 100%. Values are the means ± SEM of triplicate cultures from two separate experiments. The points without the standard error bars indicate that their individual SEMs are within the symbols. Key: (*) $P < 0.05$ as compared with controls by Fisher PLSD and unpaired Student's t-test.

progesterone and hCG, and have been used as a model of first trimester trophoblasts [19, 20].

The present study supports the feasibility of using BeWo and JEG-3 cells as a placental system to investigate the potential toxicity of environmental chemicals. Both BeWo and JEG-3 cells were found to express EGF receptor mRNA transcripts of 10 and 5.6 kb and express immunoreactive EGF receptor proteins of 150–170 kDa, which is in agreement with observations in human placentas and placental cell cultures [11, 12, 14–18]. BaP treatment of both BeWo and JEG-3 cells resulted in a concentration-related decline in binding of ^{125}I-EGF, similar to previous findings in primary cultures of early gestation human placental cells exposed to BaP [14]. Moreover, Scatchard analysis indicates a loss of high-affinity EGF binding sites in choriocarcinoma cells following treatment with BaP. In this regard, concentration-dependent declines in EGF binding have been reported previously with BaP in cultured human keratinocytes [30], mouse embryo fibroblasts [29] and hepatoma cell lines [31]. In the choriocarcinoma cells, data indicate that the reduced binding of ^{125}I-EGF to whole cells is not likely due to altered internalization of cell surface receptors because EGF binding was decreased significantly at 4° when internalization is minimal. In addition, we also observed that the loss of the receptor protein following BaP treatment is not likely due to the increased degradation since no lower molecular weight bands appeared in association with the decrease in native 150–170 kDa receptor protein. These results further support our previous finding that the smoking-related defective placental EGF receptor autophosphorylation appeared to be due to the loss of EGF receptor protein [12].

In the present study, choriocarcinoma cells responded to BaP directly with the induction of CYP1A1, which is consistent with our previous observations in human pla-

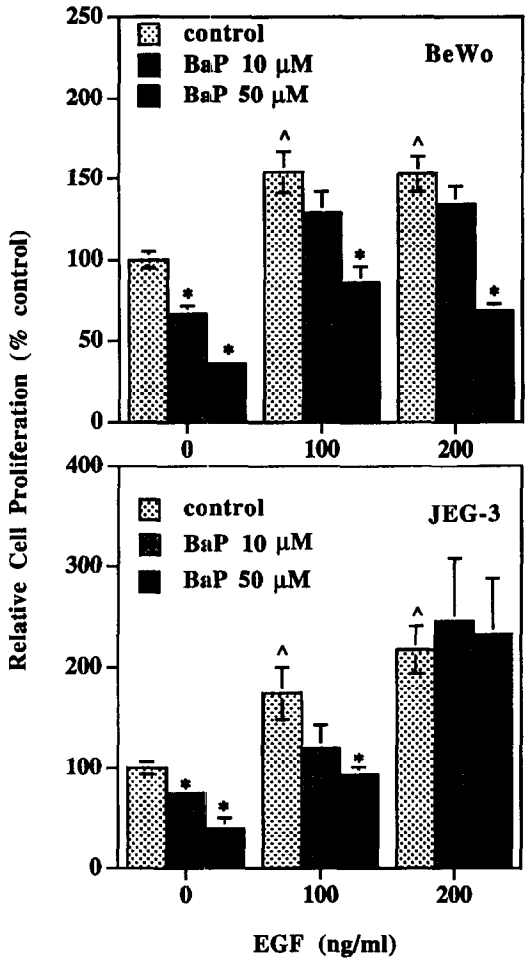

Fig. 6. Modulation by BaP of EGF-stimulated cell proliferation in BeWo and JEG-3 cells. BeWo and JEG-3 cells were cultured at densities of 5×10^3 and 2.5×10^3 cells/well, respectively, for 20 hr and treated with BaP and/or EGF for another 48 hr in the absence of serum. Values are the means ± SEM of triplicate cultures from two separate experiments. Key: (^) differs from control cells (0 EGF, 0 BaP), $P < 0.05$; and (*) differs from control (0 BaP) in each group, $P < 0.05$ by Fisher PLSD and unpaired Student's *t*-test.

cental cells in primary culture [14]. With regard to exposure, induction of CYP1A1 is observed at 1 μM BaP (250 ng/mL), an amount of BaP which is in the range reported to be present in the smoke of ten to twenty-five cigarettes [32]. Several known inducers of CYP1A1, including TCDD, have been shown to decrease EGF binding in cultured cell lines *in vitro* [29–31] and tissues in rats and inbred strains of mice *in vivo* [33–35]. In mouse fibroblasts, Ivanovic and Weinstein [29] found that exposure of BaP led to a time- and concentration-dependent decrease of EGF binding, whereas the highly reactive electrophilic metabolite of BaP, BaP-7,8-diol-9,10-oxide, did not alter EGF binding significantly. A comparison of a series of sixteen compounds further showed a correlation between the capacities to inhibit EGF binding and published data on the apparent affinities of the same compounds for the Ah receptor, as well as their capacities to induce the CYP1A1 system [29]. In this regard, Lin *et al.* [35] have reported that the Ah locus mediates the effects of TCDD on the hepatic EGF

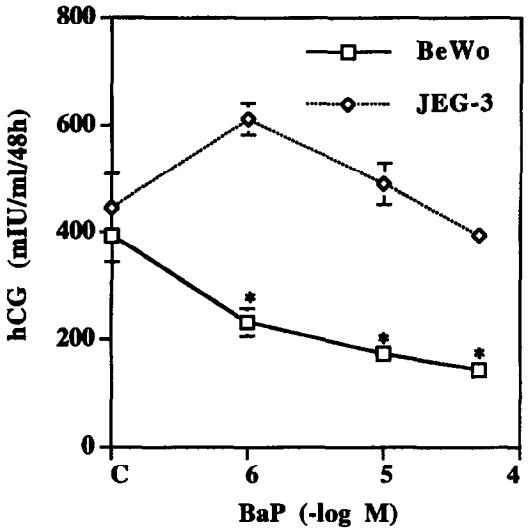

Fig. 7. Differential effects of BaP on hCG secretion by BeWo and JEG-3 cells. The cells were exposed to BeWo for 48 hr in the presence of serum. The medium was then collected for assay of hCG levels. Values are the means ± SEM of six determinations from triplicate cultures. These experiments were repeated twice with similar results. Key: (*) $P < 0.05$ as compared with controls (C) by Fisher PLSD and unpaired Student's *t*-test.

receptor in C57BL/6J mice. It warrants note, however, that TCDD has also been reported to stimulate EGF receptor expression and proliferation in the embryonic palate and ureter epithelial cells, which may be unique to early development [36, 37].

The steady-state level of mRNA for EGF receptors

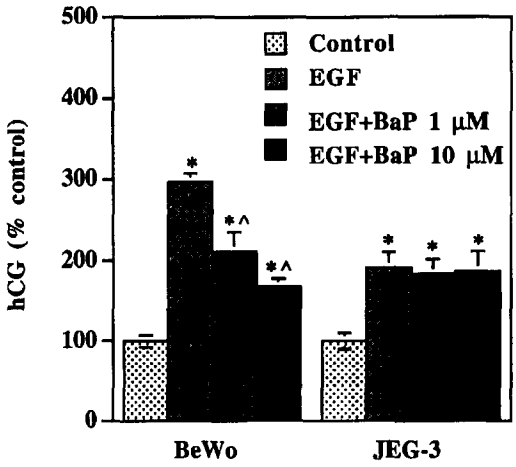

Fig. 8. Differential effects of BaP pretreatment on EGF-stimulated hCG secretion by BeWo and JEG-3 cells. The cells were treated with or without BaP for the first 24 hr in serum-containing medium and then 24 hr in serum-free medium. After being washed three times with Hanks' solution, the cells were exposed to serum-free medium with or without EGF (100 ng/mL, 17 nM) for 24 hr. hCG secretion of control BeWo (123.1 ± 9.2 mIU/mL/24 hr) and JEG-3 (682.5 ± 67.7 mIU/mL/24 hr) cells was set as 100%. Values are the means ± SEM of six determinations from triplicate cultures. Key: (*) $P < 0.05$ as compared with untreated (0 EGF, 0 BaP) controls; and (^) $P < 0.05$ as compared with EGF alone by Fisher PLSD and unpaired Student's *t*-test.

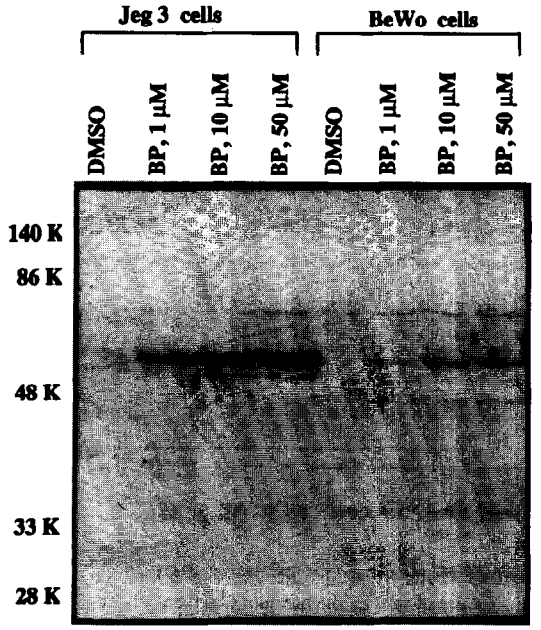

Fig. 9. Induction of CYP1A1 in BeWo and JEG-3 cells by BaP, as shown by western blot. The cells were incubated with BaP for 48 hr. One hundred micrograms protein of the whole cell lysate was electrophoresed on a 10% SDS–PAGE, transferred, and immunostained with CYP1A1 antibody (1:500), as described in Materials and Methods.

was not found to be altered significantly by BaP exposure in the BeWo and JEG-3 choriocarcinoma cell lines. In contrast, the EGF receptor and its mRNA levels were reported to be decreased in intrauterine growth-retarded and diabetes mellitus-complicated placentas [17]. TCDD induced a reduction in both EGF binding and EGF receptor mRNA steady-state levels in rat uterus [34]. However, exposure of human keratinocytes *in vitro* to TCDD and livers of TCDD-treated mice showed a reduction in maximal EGF binding without a change in the amount of mRNA for the EGF receptor [35]. The observation that TCDD induces expression of TGF-α in keratinocytes [38] led to the hypothesis that increased expression of this peptide growth factor may lead to internalization of the EGF receptor and activation of cell responses. In recent studies, however, TGF-α mRNA was not found to be increased in rat liver by TCDD [39], whereas EGF receptor mRNA was reported to be decreased in correlation with the loss of EGF binding [40]. Thus, data suggest that the effect of BaP or TCDD on EGF receptor mRNA levels may be a species- or tissue-specific response.

The mitogenic response of choriocarcinoma cells to EGF can be observed under serum-free conditions, which is in agreement with the mitogenic action of EGF or TGF-α on cytotrophoblasts [3, 4]. In choriocarcinoma cells, we found that BaP inhibited both basal and EGF-stimulated cell proliferation under serum-free conditions. The inhibition of BaP on EGF stimulated proliferation in BeWo cells does not appear to be specific, since the relative percent decrease in both groups was the same and no significant interaction of BaP and EGF was observed. On the other hand, there was a significant interaction between BaP and EGF on JEG-3 cell proliferation, suggesting that different mechanisms may be involved in the regulation of BeWo and JEG-3 cell proliferation. In a human keratinocyte cell line, Hudson *et al.* [30] found that the reduction in EGF binding correlated with a decrease in EGF-stimulated DNA synthesis, although TCDD itself stimulated DNA synthesis. Biegel and Safe [41] reported that TCDD inhibited basal and estradiol-stimulated cell proliferation of estrogen-responsive MCF-7 human breast cancer cells. In choriocarcinoma cells, we also noted that EGF at a high concentration (200 ng/mL) provides a protective effect on maintaining JEG-3 cell viability in the presence of 50 μM BaP, as was observed with serum.

The present study found that the loss of EGF receptors in choriocarcinoma cells is correlated with decreased basal and EGF-stimulated hCG secretion in BeWo cells, but not in JEG-3 cells. In this regard, recent studies with human extravillous trophoblasts in explant culture have observed significant gestation-specific differences in the ability of EGF to stimulate hCG production during early pregnancy [4, 42]. Our earlier study [14] found that BaP exposure inhibited EGF binding in human placental cell cultures from first trimester, but not term placentas. Thus, evidence indicates that developmental windows exist in placental and trophoblast development for altered responses to EGF and BaP toxicity. The differential effect of BaP on hCG secretion observed in JEG-3 compared with BeWo cells may reflect developmental differences in the state of differentiation of these two cell lines, the former being more proliferative and growing in lower concentrations of serum. EGF induces differentiation of cytotrophoblasts to form syncytiotrophoblasts and to increase hCG secretion in term placenta [6], with hCG being able to act as an autocrine regulator of further differentiation of cytotrophoblasts [43] by enhancing the gap junctional communication between trophoblasts [44]. Thus, our finding that BaP inhibited both basal and EGF-stimulated hCG secretion by BeWo cells suggests that differentiation in this cell line may be adversely affected by BaP. In addition, different mechanisms may be involved in the regulation of hCG secretion in these two cell lines, as has been reported recently for JAR human choriocarcinoma cells [45].

In conclusion, BeWo and JEG-3 cells respond to BaP directly with a decrease in EGF receptor binding and protein, alterations in cell growth, and induction of CYP1A1. In addition, these changes are associated with altered hCG secretion in BeWo cells, but not in JEG-3 cells. This study supports the feasibility of using BeWo and JEG-3 cells to investigate biomarkers of placental toxicity. This approach further serves to identify mechanisms by which individual constituents of cigarette smoke may directly alter placental growth and endocrine function and interfere with fetoplacental growth.

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